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Enhanced Generation of Monocyte Tissue Factor and Increased Plasma Prothrombin Fragment$_{1+2}$ Levels in Patients With Polycythemia Vera: Mechanism of Activation of Blood Coagulation

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Polycythemia vera (PV) is associated with a high incidence of thrombosis. The association of apparent and secondary polycythemia with thrombosis is not clear. It was suggested that activation of the coagulation system contributes to thrombus formation in PV. However, the mechanism of activation is unknown. Monocytes generate a potent tissue factor (TF) upon stimulation with various substances, which is involved in thrombus formation in various disorders. Therefore, we studied the possibility that the factor is involved in the activation of coagulation and thrombus formation also in PV. Unstimulated peripheral blood mononuclear cells (PBMC) from each of the different types of polycythemia expressed weak TF activity (2 U) and antigen (41.4 to 52.9 pg/ml), which were similar to normal controls. Following stimulation with endotoxin, PBMC from normal controls and from apparent and secondary polycythemia showed a 3.9- to 4.5-fold increase in TF, while cells from PV showed a 21-fold increase ($P < 0.001$). Similar levels were generated by PBMC after treatment of PV and at the spent phase. TF was generated by monocytes but not by lymphocytes. Plasma prothrombin fragment$_{1+2}$ (F_{1+2}) levels, assayed at the same time, were significantly higher in PV (2.46 nm) compared to normals and apparent and secondary polycythemia (0.22 to 0.32 nm), and were in a significant correlation with monocyte TF activity and antigen levels (r = 0.77, 0.87). The high levels of F_{1+2} confirm that the coagulation system is activated in PV. The increased capacity of monocytes to generate TF may be responsible for the activation of the coagulation system and thrombus formation. The hypercoagulability state that is induced by this mechanism suggests that long-life oral anticoagulation should be considered once thrombosis has been developed in PV. Am. J. Hematol. 56:5–11, 1997.

Key words: monocyte; polycythemia; polycythemia vera; thrombosis; thromboplastin (tissue factor)

INTRODUCTION

Thromboembolism is a major complication of polycythemia vera (PV) [1–3]. The association of apparent and secondary polycythemia with thromboembolism is more tenuous [4,5]. The major factor leading to thrombus formation in PV is elevation of blood viscosity [6,7]. However, thromboembolism often continues to be a major clinical problem after hematologic control has been achieved [1–3], indicating that other mechanisms are involved in thrombus formation in this disease.

Several investigators have shown that patients with PV have chronic activation of the coagulation system, suggesting that this may be one of the mechanisms that contributes to thrombus formation [8,9]. However, the

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TABLE I. Clinical Data on Patients With Polycythemia*

Patients	n	Age (years)	Hb (g/dl)	Hct (%)	WBC ($\times 10^9$/L)	Monocytes (%)	Platelets ($\times 10^9$/L)	RBC mass (ml/kg)	LAP score
P vera-active[a]	20	59	18.5	56.8	15.1	4	450	42	174
		(48–74)	(16.0–21.3)	(50.2–65.0)	(8.6–23.0)	(3–16)	(250–830)	(37–48)	96–320
P vera-treated[a]	20	62	14.7	44.6	10.2	6	380	—	152
		(50–76)	(12.8–17.0)	(38.1–49.0)	(5.7–18.9)	(3–14)	(230–640)	—	(74–282)
P vera-spent[a]	6	63	9.2	27.8	14.8	4	405	—	158
		(51–75)	(8.1–10.00)	(25.0–31.0)	(9.8–17.8)	(3–11)	(250–580)	—	(69–220)
P relative	20	62	17.9	54.7	7.1	9	336	30	80
		(49–77)	(15.8–20.2)	(50.1–60.0)	(4.4–10.5)	(4–16)	(220–430)	(28–34)	(42–132)
P secondary	20	58	18.9	55.6	7.3	7	340	40	82
		(38–75)	(16.8–21.0)	(49.8–66.0)	(4.6–11.0)	(3–15)	(270–440)	(36–50)	(44–134)
Normal controls	20	46	13.8	42.4	6.3	10	312	32[b]	78[b]
		(19–64)	(12.6–17.2)	(38.5–50.2)	(4.4–10.2)	(5–20)	(158–386)	(30–35)	(40–130)

*P, Polycythemia.
[a]The same group of patients.
[b]Normal values at Assaf Harofeh Medical Center and Hadassah University Medical Center.

pathogenesis of activation of blood coagulation is unknown.

Tissue factor (TF) is the most potent activator of the coagulation system. Monocytes and macrophages express high levels of TF after in vitro exposure to various stimuli [10,11]. Increased monocyte TF has been demonstrated in patients with various diseases that are associated with a high incidence of thromboembolism [10,11]. The monocyte TF factor may activate the coagulation system and thereby induce thrombus formation.

Prothrombin fragment$_{1+2}$ (F_{1+2}) is a peptide that is released when prothrombin is cleaved by factor Xa [12]. Elevated plasma levels of F_{1+2} are a sensitive indicator of ongoing activation of the coagulation system and are increased in patients with thrombotic and prethrombotic states [13,14].

In order to evaluate the potential role of monocyte TF in the pathogenesis of activation of the coagulation system in patients with different types of polycythemia and especially PV, we measured plasma F_{1+2} levels and simultaneous in vitro generation of monocyte TF in these patients. We found elevated levels of plasma F_{1+2} and enhanced generation of monocyte TF in patients with PV, suggesting that the factor may play an important role in activation of blood coagulation and thrombus formation in PV.

MATERIALS AND METHODS

Patients

The diagnosis of PV was based on the criteria adopted by the PV Study Group [15]. Patients in the spent phase had hemoglobin <11 g/dl for at least 1 year, which could not be attributed to treatment, bleeding, or hemolysis. Biopsies of the liver carried out in three patients revealed extramedullary hematopoiesis. All patients with spent PV were followed from the active phase of their disease. The diagnosis of apparent polycythemia was based on the combination of hematocrit >52%, normal red cell mass, normal plasma O_2 saturation, and no other disorders that may induce secondary polycythemia [5].

The clinical and laboratory characteristics of the patients at the time of the study are summarized in Table I. Fourteen patients with secondary polycythemia had chronic obstructive pulmonary disease and six had congenital cyanotic heart disease. Three patients with PV were treated with phlebotomies and seventeen with phlebotomies and hydroxyurea. Patients at the spent phase were treated with blood transfusions and occasionally with androgens and folic acid.

Isolation and Incubation of Cells

Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-hypague gradient centrifugation (Pharmacia LK13, Biotechnologies Inc., Piscataway, NJ) [16–19]. The PBMC collected from the plasma/Ficoll hypague interface were washed three times with phosphate buffered saline (PBS). Platelets were removed by two-step centrifugation, once at 140*g* and twice at 100*g* at 24°C for 10 min, and the cells were finally resuspended in RPMI 1640 supplemented with L-glutamine (Biological Industries, Beth Haemek, Israel) and 10% heat inactivated fetal calf serum (FCS) (Gibco, Paisley, Scotland) at a concentration of 3×10^6 cells/ml. The PBMC comprised 67 to 88% lymphocytes (mean 84%) and 12 to 33% monocytes (mean 16%) as assessed by light microscopy, positive staining with fluoride-inhibited nonspecific esterases (Sigma Chemical Co., St. Louis, MO), phagocytosis of at least four latex particles, and anti CD14 antibodies. The number of monocytes that had been isolated from normal controls and the different

types of polycythemia were similar. The cell suspensions contained less than 2 platelets per mononuclear cell, and less than 10 platelets per monocyte.

PBMC at 3×10^6/ml in RPM 1640 L-glutamine and 10% FCS were incubated with and without 10 μg/ml endotoxin (*Escherichia coli* 026:138, Sigma Chemical Co.) for 16 to 18 hr. After incubation, the cells were washed twice in veronal acetate buffered saline, pH 7.35, centrifuged and the pellet was stored at −70°C until monocyte TF was assayed.

The viability of the cells as assayed by the trypan blue exclusion was greater than 90% after 16 to 18 hr of incubation.

To obtain an enriched population of peripheral blood monocytes, PBMC at 3×10^6/ml were incubated with RPMI + 10% FCS in Petri dishes for 2 hr. The non-adherent cells (lymphocytes) were removed by washing 3 times with RPMI 1640. The adherent cells (80 to 85% monocytes and 15 to 20% lymphocytes) were incubated with and without endotoxin under the same conditions as PBMC. After incubation, the cells were scraped and handled as the PBMC until monocyte TF was assayed.

Monocyte TF Assays

Frozen cells were thawed at 37°C, resuspended in 1 ml veronal buffered saline, pH 7.35, and sonicated with ultrasonic vibrator for 60 sec in an ice bath [16–19].

TF activity assay. TF activity was measured by a modified prothombin time [16–19]: 0.1 ml pooled anticoagulated plasma from at least 10 normal donors was incubated with 0.1 ml cell suspension at 37°C for 1 min, 0.1 ml of 0.025 m $Cacl_2$ was added, and the clotting time recorded. Each sample was run in duplicates. TF activity was expressed in units calculated from standard cures of the logarithm of the activity of serial dilution of standard thromboplastin (Baxter Healthcare Corp., Miami, FL). Dilutations of 1:1024 thromboplastin (170 sec) and 1:16 (32 sec) were equal to 1 and 64 U, respectively.

TF antigen assay. TF antigen was measured with the Imubind Tissue Factor ELISA Kit (American Diagnostica, Greenwich, CT), according to the manufacturer's recommendations. The results were calculated with a standard curve from 0 to 1,000 ng/ml of human TF provided with the kit.

F_{1+2} Assay

Concomitantly with the collection of blood for PBMC isolation, blood was drawn in heparin from the same patients, centrifuged immediately, treated with sample treatment reagent, and stored at −70°C. F_{1+2} was assayed with Prothrombin $Fragment_{1+2}$ ELISA (Organon Teknika, Durham, WI). The results were calculated using a standard curve from 0 to 10 nM of F_{1+2} provided with the kit.

TABLE II. TF Activity in PBMC From Patients With Polycythemia*

Patients	No.	TF activity (U)		*P*
		−	+	
Normals	20	1.9 ± 0.4 (1.2–2.6)	7.5 ± 2.0 (4–11)	
Apparent polycythemia	20	1.9 ± 0.4 (1.3–2.5)	8.5 ± 2.4 (4–12)	
Secondary polycythemia	20	1.9 ± 0.2 (1.2–2.4)	8.3 ± 2.3 (5–12)	
PV active[a]	20	2.0 ± 0.4 (1.5–2.6)	41.5 ± 9.0 (26–58)	<0.001
PV treated[a]	20	2.0 ± 0.3 (1.4–2.5)	40.0 ± 6.0 (30–52)	<0.001
PV spent[a]	6	1.9 ± 0.3 (1.4–2.2)	41.5 ± 7.8 (27–49)	<0.001

*Isolated PBMC were incubated without (−) and with (+) 10 μg/ml endotoxin for 16–18 hours and TF activity was determined as described in Materials and Methods. The results are mean ± SD (range).
[a]The same group of patients.

Statistical analysis was performed using the paired Student's *t*-test.

RESULTS

TF Activity and Antigen Levels in Endotoxin-Stimulated and Unstimulated Cells

The TF levels measured in PBMC from normal controls and from patients with PV and apparent and secondary polycythemia are presented in Tables II and III. The unstimulated cells from normal controls and patients with each of the different types of polycythemia expressed weak TF (activity 1.9 to 2.0 U; antigen 41.4 ± 7.9 to 52.9 ± 13.2 pg/ml). The mean TF activity and antigen was slightly higher in the unstimulated PBMC from patients with PV at the active stage and after treatment (2 U; 50.9 and 50.3 pg/ml) in comparison to other types of polycythemia (1.9 U; 44 and 41 pg/ml). However, the differences were not statistically significant ($P > 0.1$). After stimulation of PBMC with 10 ng/ml endotoxin for 16 to 18 hr, TF activity and antigen levels were elevated to the same extent in cells from normal controls and from patients with apparent and secondary polycythemia (7.5 ± 2.0 to 8.5 ± 2.4 U; 362.5 ± 80.2 to 367.5 ± 88.7 pg/ml). However, PBMC from patients with PV at the active stage of the disease, which were stimulated with endotoxin under the same conditions, generated a significantly higher TF levels (activity 41.5 ± 9 U; antigen 1,157.5 ± 208.7 pg/ml) in comparison to normal controls and patients with apparent and secondary polycythemia ($P < 0.001$). A similar increase of TF levels was demonstrated in PV after cytoreductive treatment and at the spent stage of the disease (activity 40 ± 6 and 41.5 ± 7.8 U; antigen 1,153 ± 172 and 1,020 ± 133 pg/ml, respectively). The same results were obtained when TF was

TABLE III. TF Antigen Levels in PBMC From Patients With Polycythemia*

Patients	No.	TF antigen (pg/ml) −	TF antigen (pg/ml) +	*P*
Normals	20	52.9 ± 13.2 (30–84)	364.9 ± 72.0 (240–480)	
Apparent polycythemia	20	44.0 ± 9.0 (30–65)	367.5 ± 88.7 (180–510)	
Secondary polycythemia	20	41.4 ± 7.9 (30–60)	362.5 ± 80.2 (260–520)	
PV active[a]	20	50.9 ± 11.8 (32–72)	1,157 ± 208.7 (740–1,530)	<0.001
PV treated[a]	20	50.3 ± 8.3 (32–60)	1,153 ± 172 (900–1,460)	<0.001
PV spent[a]	6	47.3 ± 3.3 (44–52)	1,020 ± 132.9 (960–1,210)	<0.001

*Isolated PBMC were incubated without (−) and with (+) 10 μg/ml endotoxin for 16–18 hr and TF antigen levels were assayed as described in Materials and Methods. The results are mean ± SD (range).
[a]The same patients.

calculated per single monocyte in the unstimulated and the stimulated cells (data not shown).

F_{1+2} Levels in Patients with Polycythemia

The mean levels of plasma F_{1+2} were similar in normal controls and in patients with apparent and secondary polycythemia (0.22 ± 0.13 to 0.32 ± 0.177 nM) (Table IV). The levels were significantly higher in patients with active PV (2.46 ± 1.0 nM) ($P < 0.0001$). Regression analysis showed a high correlation between TF activity and antigen levels in stimulated PBMC, and plasma F_{1+2} concentrations in patients with PV ($r = 0.77$ and 0.87, respectively) (Figs. 1 and 2).

TF in Purified Monocytes and Lymphocytes

The generation of TF was studied in purified monocyte and lymphocyte from eight patients with PV (Table V). Endotoxin-stimulation monocytes generated the same levels of TF as PBMC (activity 50 ± 1 U; antigen 1,210 ± 184 pg/ml). Endotoxin-stimulated lymphocytes expressed weak TF activity (2.0 ± 0.5 U) and antigen (46 ± 6 pg/ml).

DISCUSSION

Polycythemia vera is associated with high incidence of thromboembolic complications [1–3]. The association between apparent and secondary polycythemia and thromboembolic complications is not clear [4,5]. The major factor responsible for the development of thrombosis is the increased blood viscosity, directly related to the hematocrit volume [6,7]. However, other mechanisms may be involved in the development of thrombosis in PV.

TABLE IV. Plasma F_{1+2} Levels in Patients With Polycythemia

Patients	No.	F_{1+2} (nM)	*P*
Normals	20	0.27 ± 0.20 (0–0.54)	
Apparent polycythemia	15	0.22 ± 0.13 (0–0.52)	
Secondary polycythemia	16	0.32 ± 0.17 (0.10–0.60)	
PV active	13	2.46 ± 1.00 (1.10–4.30)	<0.001

Several studies have suggested that the coagulation system is activated in PV. This is based on abnormalities of the coagulation system such as reduced levels of factor V, protein C, protein S, and antithrombin III [20,21], high fibrinolytic activity [20], and increased levels of β_2 thromboglobulin and fibrinopeptide A [8]. However, the mechanism that triggers the activation of the coagulation system is unknown.

Monocytes generate a potent TF after stimulation with various substances including endotoxin [22], tuftsin [18], anticardiolipin antibodies [19], tumor necrosis factor [23], complement [24], antigen antibody complexes [25], and others [10,11]. There is substantial evidence suggesting that monocyte TF plays a prominent role in activation of the coagulation system and clot formation: (1) Increased TF levels were demonstrated in monocytes from patients with a high incidence of thromboembolic complications such as bacterial sepsis with disseminated intra-vascular coagulation (DIC) [10,11], cancer [26], inflammatory bowel disease [27], pregnancy-related complications, and other [10,11]. The patients with the highest monocyte TF levels had the greatest incidence of thromboembolic phenomena [28]; (2) Fibrin had been identified on the surface of endotoxin-activated monocytes [29]; (3) Increased TF activity was found in monocytes and macrophages adjacent to areas of fibrin deposits in tissues of patients with inflammatory and immunological disorders [10,11]; (4) Animals rendered leukopenic failed to develop DIC in response to endotoxin injections and fibrin could not be detected in their renal glumeruli [30].

In the present study, we made several observations that link monocyte TF and activation of the coagulation systems in PV: (1) Plasma F_{1+2}, a sensitive marker of activation of the coagulation system [12–14], is significantly increased in PV and is normal in apparent and secondary polycythemia. This finding confirms previous reports about the activation of the coagulation system in PV [8,20,21]; (2) Unstimulated monocytes from normal controls and from each of the different types of polycythemia expressed weak TF. However, the mean value of TF activity and antigen levels was slightly higher in PV in comparison to apparent and secondary polycythemia. Following stimulation of the monocytes from normal

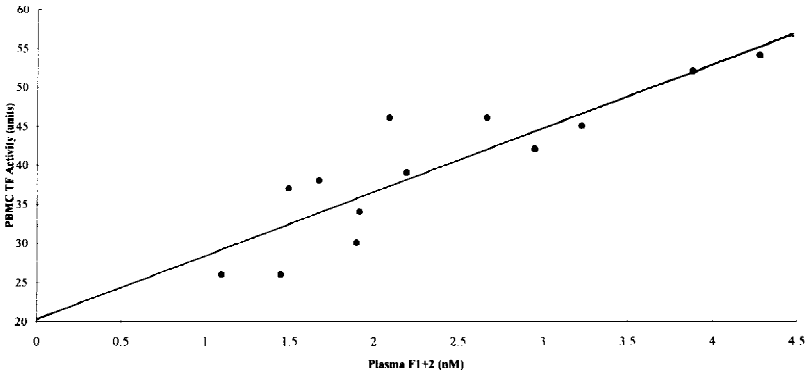

Fig. 1. The correlation between PBMC TF activity and plasma F_{1+2} levels in PV. Cells were incubated with 10 μg/ml endotoxin for 16–18 hr and TF activity was determined as described in Materials and Methods (r = 0.77).

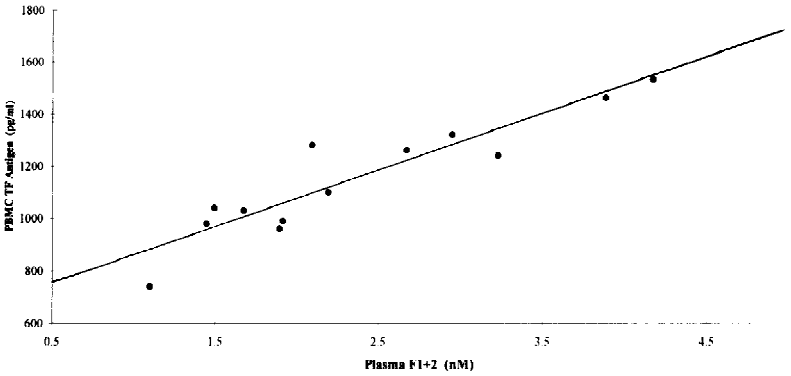

Fig. 2. The correlation between PBMC TF antigen levels and plasma F_{1+2} levels in PV. Cells were incubated with 10 μg/ml endotoxin for 16–18 hr and TF activity was assayed as described in Materials and Methods (r = 0.87).

TABLE V. Cell of Origin of TF in PV*

Cells	n	TF activity (U)	TF antigen (pg/ml)	*P*
Normal PBMC	20	42 ± 9	1,158 ± 209	<0.001
PV, monocytes[a]	8	50 ± 14	1,210 ± 184	<0.001
PV, lymphocytes[a]	8	2 ± 0.5	46 ± 6	

*Cells were separated and TF was assayed after 16–18 hr of incubation with 10 μg/ml endotoxin as described in Materials and Methods.
[a]The same patients.

controls and from patients with apparent and secondary polycythemia with endotoxin, the cells generated 3.9 to 4.5 times more TF compared to the unstimulated cells. However, the endotoxin-stimulated monocytes from patients with PV showed a 21-fold increase in TF levels compared to unstimulated monocytes, and a 5.5-fold increase compared to stimulated monocytes from normal controls and apparent and secondary polycythemia ($P < 0.001$). Similar results, namely normal levels of TF in unstimulated monocytes and a significant increase in TF following stimulation of the cells had been shown also in other diseases associated with thrombus formation such as Hodgkin's disease [31], toxemia of pregnancy [32], and Crohn's disease [27]; (3) There is a significant correlation between TF activity and antigen in the stimulated monocytes and plasma F_{1+2} in PV ($r = 0.77$ and 0.87, respectively). When combining all these findings, it is possible that stimulation of monocytes in patients with PV by endotoxin during episodes of infections, or by other substances such as immune complexes [25], interleukins [23], and complement [24], which induce monocyte TF by the same mechanism as endotoxin, induce a potent TF in monocytes. The monocyte TF may activate the coagulation system and lead to thrombus formation.

In the present study we found that the coagulation system in patients with PV is activated in vivo, while a significant increase in TF was found only in vitro in the stimulated cells. Similar results had been demonstrated in patients with Crohn's disease [27], using fibrinopeptide A as a marker of activation of the coagulation system. The slight increase of monocyte TF in the unstimulated cells in these patients in comparison to apparent and secondary polycythemia, can contribute to some extent to the in vivo activation of the coagulation system. However, it cannot be fully responsible for the activation of coagulation and the significant correlation between TF in stimulated monocytes and the increased plasma F1.2 in patients with PV. Therefore, we have to assume that the activation of the coagulation system is more complex and involves other mechanisms that act synergistically with the monocyte TF. Other mechanisms that have been described include thrombocytosis [3], activated platelets [8], decreased tissue plasminagen activator [33], increased fibrinogen level [34], activated protein C resistance and reduced levels of protein C, protein S, and antithrombin III [21].

We did not have enough information about the thromboemboli complications in our patients, and, therefore,

we could not correlate between monocyte TF levels and thrombosis in patients with PV. However, the hypercoagulable state that is induced by the increased levels of monocyte TF suggests that long-life anticoagulation is indicated once thrombosis has developed in patients with PV.

It was shown that autologus platelets may stimulate monocytes TF even without an additional stimulus [35]. This effect was evident at platelet/monocyte ratio varying from 15 to 300. The platelet/monocyte ratio in the present study was less than 10. Therefore, it is unlikely that the platelets played a significant role in TF generation in PV. However, patients with PV at diagnosis had thrombocytosis and it may contribute to TF generation in unstimulated monocytes.

In the present study, we made several additional observations: (4) TF was generated in monocytes and not in lymphocytes as was described previously [36]; (5) The monocytes from patients with PV retained the capacity to generate TF even after treatment and at the spent phase of the disease. Other characteristics of PV, such as high leukocyte alkaline phosphotase scoring [1] and increased sensitivity of erythroid progenitors to erythropoietin [37], also persist after treatment and at the spent phase; (6) The association between apparent and secondary polycythemia and thrombotic disease is not clear [4,5]. It is, therefore, of interest that monocyte TF is not increased in these disorders. The different response of the endotoxin-stimulated monocytes may serve as a useful tool in discriminating PV from other types of polycythemia.

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